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Mapping of RNA⁻ Temperature-Sensitive Mutants of Sindbis Virus: Complementation Group F Mutants Have Lesions in nsP4

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Temperature-sensitive (*ts*) mutants of Sindbis virus belonging to complementation group F, *ts*6, *ts*110, and *ts*118, are defective in RNA synthesis at the nonpermissive temperature. cDNA clones of these group F mutants, as well as of ts^+ revertants, have been constructed. To assign the *ts* phenotype to a specific region in the viral genome, restriction fragments from the mutant cDNA clones were used to replace the corresponding regions of the full-length clone Toto1101 of Sindbis virus. These hybrid plasmids were transcribed in vitro by SP6 RNA polymerase to produce infectious transcripts, and the virus recovered was tested for temperature sensitivity. After the *ts* lesion of each mutant was mapped to a specific region of 400 to 800 nucleotides by this approach, this region of the cDNA clones of both the *ts* mutant and ts^+ revertants was sequenced in order to determine the precise nucleotide change and amino acid substitution responsible for each mutation. Rescued mutants, which have a uniform background except for one or two defined changes, were examined for viral RNA synthesis and complementation to show that the phenotypes observed were the result of the mutations mapped. *ts*6 and *ts*110 had a single base substitution in nsP4, resulting in replacement of Gly by Glu at position 153 or position 324, respectively. It is of interest that nsP4 contains the Gly-Asp-Asp motif characteristic of a number of viral replicases, and this, together with the fact that all RNA synthesis in *ts*6-infected cells and, to a lesser extent, in *ts*110-infected cells shut off when the cells were shifted from a permissive to a nonpermissive temperature, suggests that nsP4 is the virus polymerase. *ts*118 was a double mutant. It contained a single base substitution in nsP2, resulting in replacement of Val by Ala at position 425 that resulted in the formation of minute plaques, but not in a reduction in the plaque number at the nonpermissive condition. The second change, a substitution of Gln by Arg in *ts*118 at residue 93 in nsP4, had little apparent phenotype on its own, but in combination with the change in nsP2 led to a *ts* phenotype. Thus, in each case the mutation responsible for the temperature sensitivity of the three known complementation group F mutants lay in nsP4. In addition, the result with *ts*118 suggests that nsP2 and nsP4 may interact with each other in a complex.

Sindbis virus is a well-studied member of the alphavirus family. Its genome is a single molecule of plus-strand RNA 11,703 nucleotides in length that is capped at the 5′ end and polyadenylated at the 3′ end (35). During replication, the parental 49S plus-strand RNA is transcribed into a complementary minus strand which serves as a template for the synthesis of both 49S plus-strand genomic RNA and a 26S subgenomic RNA. Nonstructural polypeptides are translated from the genomic 49S RNA as two polyprotein precursors that are processed by cotranslational or posttranslational cleavage into four nonstructural proteins, called nsP1, nsP2, nsP3, and nsP4, which are required for RNA replication (11). Three structural polypeptides are produced by processing of a polyprotein precursor translated from the subgenomic 26S mRNA.

Large numbers of temperature-sensitive (*ts*) mutants of the HR strain of Sindbis virus have been isolated and characterized (3, 30, 34). Mutants may be defective in RNA replication (RNA^- mutants) or in the production of the structural proteins (RNA^+ mutants) and have been grouped by complementation into four RNA^- groups (A, B, F, and G) and three RNA^+ groups (C, D, and E) (4, 30). Representative mutant-revertant pairs from RNA^+ groups have been analyzed by sequence analysis, and there is an excellent correlation between specific nucleotide changes and phenotypes (1, 10, 15). None of the RNA^- mutant defects has been rigorously assigned to specific nonstructural proteins or RNA sequences. These mutants presumably contain *ts* lesions in the viral nonstructural proteins which function to replicate viral RNA.

A full-length cDNA clone of Sindbis virus has been constructed that can be transcribed in vitro by SP6 RNA polymerase to produce infectious full-length transcripts (22). Viruses produced from in vitro transcripts are identical to Sindbis virus and show strain-specific phenotypes reflecting the source of RNA used for cDNA synthesis (17, 22). This full-length clone can be used to study interesting phenotypes of Sindbis virus.

We have used this approach to define precisely the mutations responsible for the *ts* phenotypes of Sindbis virus complementation group F mutants. Mutants *ts*6, *ts*110, and *ts*118 of complementation group F are defective in RNA synthesis at the nonpermissive temperature. The best-characterized member, *ts*6, ceases all viral RNA synthesis after a shift from permissive to nonpermissive conditions, and it has been postulated that *ts*6 has a defect in the elongation activity of the replicase (2, 14, 26). cDNA clones of these mutants, as well as of ts^+ revertants, have been constructed, and restriction fragments from the mutant cDNA clones were used to replace the corresponding regions of a full-length clone of Sindbis virus. These plasmids were transcribed in vitro by SP6 RNA polymerase to produce infectious transcripts, which were then tested for *ts* phenotype. The viruses recovered from these transcripts have been

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characterized. Together with sequence analysis of the cDNA clones, these experiments have defined the mutations responsible for the group F mutants.

MATERIALS AND METHODS

Virus stocks, growth, and purification. Mutant *ts*6, obtained originally from B. Burge, was isolated from the HR strain of Sindbis virus following mutagenesis with nitrosoguanidine (3). Mutants *ts*110 and *ts*118 were isolated from a small-plaque strain of HR Sindbis virus following mutagenesis with nitrous acid (30). Revertants were isolated by plaquing mutant stocks at 30 and 40°C. A single virus plaque of a ts^+ revertant was picked from the 40°C plate, and the virus was eluted into 1 ml of Eagle medium containing 5% fetal calf serum. This revertant plaque was used to infect primary chicken cells at 40°C, and the resulting stocks, following plaque assay at 30 and 40°C, were used as infecting stocks for RNA preparation. Viruses were grown in primary or secondary chicken embryo fibroblasts and harvested 10 to 20 h after infection, depending on the mutant. Mutants and revertants were grown at 30 or 40°C, respectively. Viral RNA was isolated as described before (24).

cDNA cloning. cDNA synthesis for *ts*6, *ts*110, and *ts*118, as well as for their revertants, followed the procedure of Okayama and Berg (20). A primer complementary to a sequence near the start codon of capsid protein (nucleotides [nt] 7642 to 7661 of the genomic RNA) was made; this primer also contained the recognition site for *Xba*I restriction endonuclease at its 5′ end. This primer was used for first-strand synthesis, and second-strand synthesis was done with *Escherichia coli* DNA polymerase I, *E. coli* RNase H, and *E. coli* DNA ligase. Phosphorylated *Eco*RI linkers were ligated to the double-stranded cDNA to facilitate later digestion with *Xba*I (see below). The double-stranded cDNA was divided into two portions for cloning. For the 5′ library, the *Acc*I (nt83)-*Sac*II (nt2771) fragment of the cDNA, which encodes nsP1 and the N-terminal half of nsP2, was cloned into Kahn5. Kahn5 is a plasmid containing a cDNA copy of the 5′ terminus of the Sindbis virus genome in Proteus 1, a vector consisting of the replicon and β-lactamase genes of pBR322 and an SP6 RNA polymerase promoter (22; H. V. Huang and C. M. Rice, unpublished). The 3′ library was constructed by cloning the *Bgl*II (nt2268)-*Xba*I (nt7662) fragment of the cDNA, encoding the C-terminal half of nsP2 and all of nsP3 and nsP4, into plasmid pMT21, an ampicillin-resistant cloning vector derived from pBR322 (the *Xba*I site is not present in this viral RNA but was introduced by the primer, as noted above).

Construction of hybrid genomes. Hybrid genomes were produced by replacing restriction fragments in Sindbis virus clone Toto1101 (22) with the corresponding regions from cDNA clones derived from the mutants or their revertants (17). Details of restriction sites used are included in the figure legends. Full-length hybrid plasmids that contained one of three nonoverlapping intervals (A, B, and C) from the mutants substituted into Toto1101 were first constructed for gross mapping. Plasmids with interval A contained the sequence of the mutant from the *Ssp*I (nt504) to the *Cla*I (nt2713) site in Toto1101. (Toto1101 contains approximately 13,638 nucleotides; numbering begins from the first nucleotide of the Sindbis virus genome.) Since the *Ssp*I site is not unique, a shuttle vector, πnsP12 (S. A. Chervitz and C. M. Rice, unpublished), containing the *Sac*I (nt13552; a site in the vector upstream of the SP6 promoter) to *Eco*RV (nt2750) region of Toto1101 cloned in πAN7 (18) was digested with *Ssp*I (nt504) and *Cla*I (nt2713) and ligated with the corresponding fragment of 5′ cDNA of the *ts* mutant. The *Sac*I-*Cla*I fragment of the resulting clone was then cloned into Toto1101 which had been cut with *Sac*I and *Cla*I and treated with calf intestinal alkaline phosphatase. Interval B plasmids contained the sequence of the mutant from *Cla*I (nt2713) to *Spe*I (nt5262) in Toto1101 and was constructed by replacing this fragment in Toto1101 with the corresponding fragment from the 3′ cDNA library of the *ts* mutant. Interval region C plasmids contained the *Spe*I (nt5262) to *Aat*II (nt7999) region of the *ts* mutant in Toto1101 and was constructed by digesting the 3′ cDNA library of the *ts* mutant with *Spe*I (nt5262) and *Bam*HI (nt7334) and cloning into shuttle vector πnsP34, which is a πAN7 derivative containing the *Pvu*II (nt5160) to *Nco*I (nt8038) fragment of Toto1101 (obtained from H. V. Huang). The *Spe*I to *Aat*II fragment of the resulting clones was then used to replace the corresponding fragment of Toto1101.

For fine mapping of the B region, plasmids were constructed that contained three overlapping subregions referred to as B1, B2, and B3. Subregions B1, covering the region *Cla*I (nt2713)-*Avr*II (nt4280), and B3, covering the *Avr*II (nt4280)-*Spe*I (nt5262) region, were cloned directly into Toto1101. Subregion B2, covering the *Ava*I (nt3546)-*Bam*HI (nt4633) region, was obtained from the shuttle vector Kahn5B, consisting of the *Cla*I (nt2713) to *Eco*RI (nt5869) fragment of Sindbis virus subcloned into Kahn5. Three clones containing overlapping subregions C1, C2, and C3 were constructed for fine mapping of the C region with πnsP34. Fragment *Spe*I (nt5262)-*Hin*dIII (nt6267), *Pst*I (nt5824)-*Hpa*I (nt6919), or *Nsi*I (nt6461)-*Bam*HI (nt7334) of the *ts* mutant was cloned into πnsP34, and the *Spe*I (nt5262)-*Aat*II (nt7999) fragment was used to replace the corresponding fragment in Toto1101.

In vitro transcription and transfection. RNA transcripts were synthesized in vitro with SP6 RNA polymerase, using supercoiled plasmid template or plasmid DNA digested with the appropriate restriction endonuclease for the production of runoff transcripts, as described previously (22). The resulting transcripts were transfected into confluent monolayers of secondary chicken cells in 35-mm multiwell tissue culture plates, and the phenotype of the recovered virus was tested. Plaques were quantitated by overlaying the monolayers with 2 ml of 1% agarose in Eagle medium containing 2% fetal calf serum, followed by incubation at 30 and 40°C. Plaques were visualized by staining with neutral red or crystal violet after incubation for 36 to 40 h at 40°C or for 60 to 72 h at 30°C.

Analysis of viral RNA synthesis. Chicken embryo fibroblast monolayers (60-mm plate) were infected with Sindbis virus HR or *ts* mutants or recombinant viruses recovered from the hybrid cDNA clones at a multiplicity of 50 PFU/cell in phosphate-buffered saline (PBS) (6) containing 1% fetal calf serum and dactinomycin (ActD) (1 μg/ml) and incubated at 30 or 40°C for 1 h. At the end of the adsorption period, the inocula were removed, and the cells were washed with warmed medium and incubated at 30 or 40°C in Eagle medium containing 3% fetal calf serum and ActD (1 μg/ml). For the shift from 30 to 40°C, at 3.5 h postinfection (p.i.) one set of 30°C plates was washed once with warmed medium lacking ActD; warmed medium containing ActD (1 μg/ml) was then added, and the plates were shifted to 40°C. At 10 h p.i. (30°C), 6 h p.i. (40°C), or 8 h p.i. (after the shift to 40°C), cells were harvested.

The amount of viral RNA present was quantitated by the cytoplasmic dot hybridization method of White and Bancroft

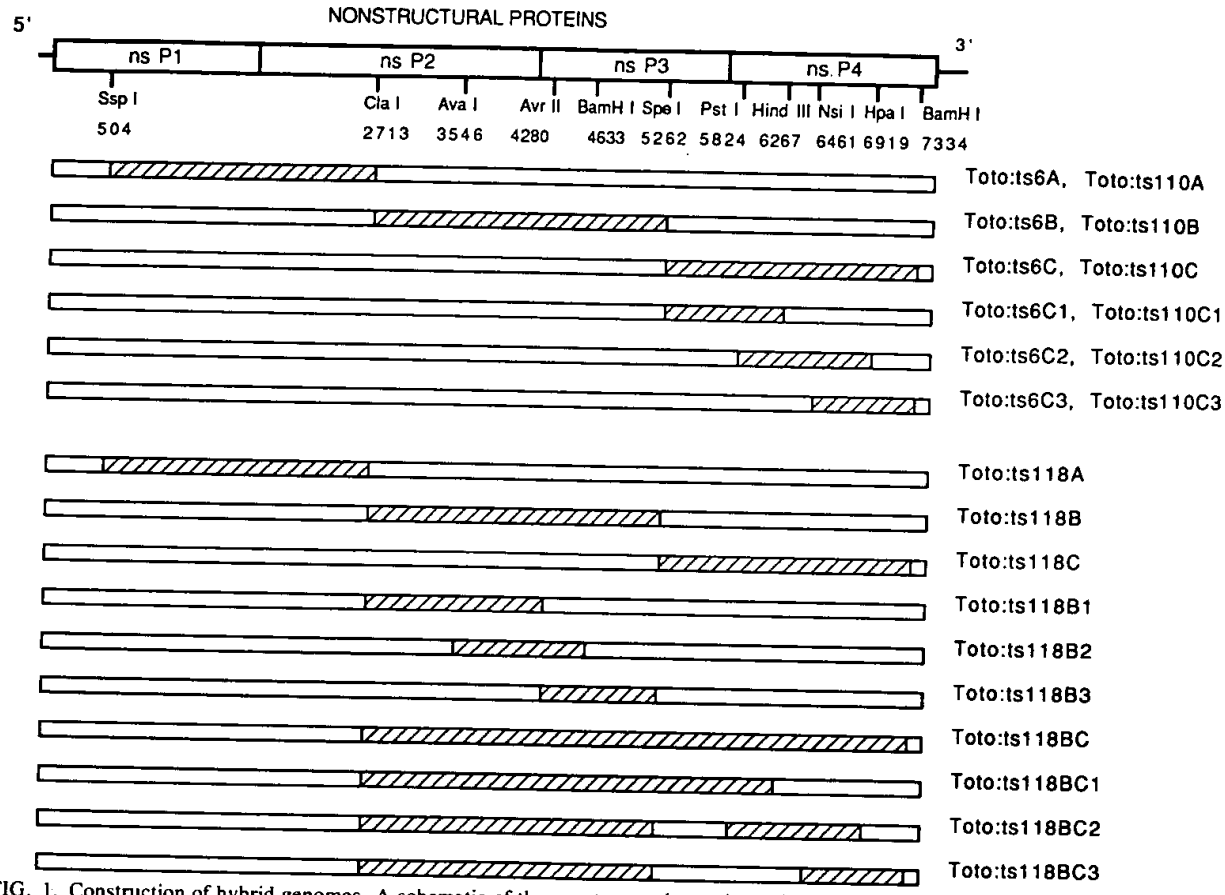

FIG. 1. Construction of hybrid genomes. A schematic of the nonstructural-protein-coding region of Sindbis virus cDNA clone Toto1101 (22) is shown together with a number of restriction sites and their positions (nt) in the Sindbis virus genome numbered from the 5' end according to Strauss et al. (33). Translated regions are shown as the open boxes, in which the names of the various proteins are indicated. Nontranslated regions are shown as a single line. The hatched boxes show the location of restriction fragments in clone Toto1101 that were replaced with the corresponding restriction fragments from the *ts* mutants or their revertants. The names used to refer to these hybrid clones are shown at the right.

(37). Equal numbers of cells were washed with cold PBS and then lysed with 1% Nonidet P-40 in TE buffer (10 mM Tris chloride [pH 7.5], 0.1 mM EDTA). Nuclei were pelleted, and the supernatant was treated with 14.8% formaldehyde in 1× SSC (150 mM sodium chloride, 15 mM sodium citrate) at 60°C for 15 min. RNA samples (10 to 20 μl) were blotted onto the nitrocellulose membranes and probed with ^{32}P-labeled minus-strand RNA from the region of the genome (the 26S region) encoding the structural proteins. This probe was transcribed with SP6 RNA polymerase from a cDNA clone of Sindbis virus that contained the structural protein region only inserted in an inverted sense downstream from an SP6 promoter. Relative amounts of RNA were determined by a beta-scanning counter. All results were corrected for the amount of incorporation into mock-infected plates, which was between 0.5 and 1% of the incorporation into cells infected with the parental strain of Sindbis virus.

Alternatively, RNA synthesis following the shift was assayed by examining the incorporation of [^{3}H]uridine into infected cells. Following infection at 30°C, cells were shifted at 3 h p.i. to 40°C and labeled with [^{3}H]uridine (20 μCi/ml) in the presence of ActD from 3.5 to 8 h p.i. Monolayers were then washed with PBS and lysed with 0.5 ml of 2% sodium dodecyl sulfate, 50 μl was precipitated with trichloroacetic acid, and the incorporated radioactivity was quantitated by liquid scintillation counting.

Complementation analysis. Complementation tests were performed as described by Strauss et al. (30) but 35-mm multiwell plates and a multiplicity of infection of 20 PFU/cell for each mutant were used. A complementation index was calculated as the yield from the mixed infection divided by the sum of the yields following infection by each parent alone. A complementation index was calculated separately for each mutant in tests in which the two mutants differed markedly in plaque size. The absolute magnitude of the complementation index is dependent on the yield of parental viruses (i.e., the extent of leakage of the parents), as complementation is always inefficient (4, 5, 30), not exceeding 1 to 10% of the wild-type yield, and in the case of *ts*118 only one-way complementation could be demonstrated because of relatively high yields of *ts*118 at 40°C.

RESULTS

Construction of recombinant plasmids. In order to localize the *ts* mutations of *ts*6, *ts*110, and *ts*118, we constructed and tested a number of recombinant plasmids. The constructs are illustrated in Fig. 1. In each case small (873 to 2,584 nt)

TABLE 1. Constructs tested for *ts* phenotype at the nonpermissive temperature

Recombinant clone[a]	Fragment replaced (nt)	Phenotype[b]	Location of mutation (nt)
Toto:*ts*6A	504–2713	wt	5824–6267
Toto:*ts*6B	2713–5262	wt	
Toto:*ts*6C	5262–7334	*ts*	
Toto:*ts*6C1	5262–6267	*ts*	
Toto:*ts*6C2	5824–6919	*ts*	
Toto:*ts*6C3	6461–7334	wt	
Toto:*ts*110A	504–2713	wt	6461–6919
Toto:*ts*110B	2713–5262	wt	
Toto:*ts*110C	5262–7334	*ts*	
Toto:*ts*110C1	5262–6267	wt	
Toto:*ts*110C2	5824–6919	*ts*	
Toto:*ts*110C3	6461–7334	*ts*	
Toto:*ts*118A	504–2713	wt	2713–3546, 5824–6267
Toto:*ts*118B	2713–5262	(*ts*)	
Toto:*ts*118B1	2713–4280	(*ts*)	
Toto:*ts*118B2	3546–4633	wt	
Toto:*ts*118B3	4280–5262	wt	
Toto:*ts*118C	5262–7334	wt	
Toto:*ts*118BC	2713–7334	*ts*	
Toto:*ts*118BC1	2713–6267	*ts*	
Toto:*ts*118BC2	2713–5262, 5824–6919	*ts*	
Toto:*ts*118BC3	2713–5262, 6461–7334	(*ts*)	

[a] See Fig. 1.
[b] RNA transcripts were transfected onto cells at 30 or 40°C, and the plaque titer was determined as described in Materials and Methods. wt, Wild type. (*ts*), Partially *ts*, in that plaque size but not plaque number is reduced at 40°C.

restriction fragments in the Sindbis virus cDNA clone Toto1101, from which infectious RNA can be transcribed in vitro with SP6 RNA polymerase (22), were replaced with cDNA from a mutant or its revertant. In this figure, the restriction sites used to construct the hybrid genomes and their numbering from the 5′ end of the RNA (33) are also shown. The genomic region encoding the nonstructural proteins was first divided into three large nonoverlapping regions, A, B, and C, for gross mapping. For fine mapping, regions B and C were each subdivided into three overlapping regions (B1, B2, and B3 and C1, C2, and C3). The 5′ 444 nt and the 3′ 265 nt of the coding region for the nonstructural proteins, which were not covered by hybrid genome constructions, were sequenced in each case to ensure that no changes had occurred within these regions.

RNA was transcribed in vitro from the recombinant plasmids with SP6 RNA polymerase and transfected onto monolayers of chicken cells. Monolayers were incubated under agarose at 30 or 40°C to determine whether the virus recovered in each case was temperature sensitive.

Localization of the mutations in *ts*6, *ts*110, and *ts*118. The results obtained with the constructs tested are summarized in Table 1. For *ts*6, of the three large interval replacement clones (Toto:*ts*6A, Toto:*ts*6B, and Toto:*ts*6C) tested, plasmids Toto:*ts*6A and Toto:*ts*6B gave rise to virus that exhibited wild-type growth at the nonpermissive temperature, while *ts* virus was obtained from plasmid Toto:*ts*6C. This localized the *ts* mutation to the interval nt5262 to nt7334 of the genome. Plasmids Toto:*ts*6C1, Toto:*ts*6C2, and Toto:*ts*6C3, containing three smaller intervals in the C region, were then constructed and tested. Plasmids Toto:*ts*6C1 and Toto:*ts*6C2 gave rise to *ts* virus, whereas Toto:*ts*6C3 did not

TABLE 2. Plaque morphology and RNA synthesis by *ts*118 at 40 and 30°C

Virus	Plaque size		Efficiency of plaquing[a] (40°C/30°C)	Relative RNA synthesis[b]		
	40°C	30°C		40°C	30°C	Shift to 40°C
Toto1101	Large	Large	2.6×10^{-1}	1.00	1.00	1.00
*ts*118	Minute	Small	5.0×10^{-5}	0.07	0.70	0.32
Toto:*ts*118B1	Minute	Small	4.0×10^{-1}	0.28	0.99	0.58
Toto:*ts*118C	Large	Large	5.0×10^{-1}	0.71	0.93	0.84
Toto:*ts*118BC	Minute	Small	5.0×10^{-5}	0.08	0.73	0.48
Toto:*ts*118BC*[c]	Minute	Small	2.6×10^{-5}	0.08	0.76	0.44
*ts*118R	Large	Large	5.4×10^{-1}	0.70	1.14	0.81

[a] Plaque titer at 40°C divided by that at 30°C.
[b] Viral RNA synthesis (relative to that by Toto1101) was assayed by dot hybridization following infection at 40 or 30°C or after a shift to 40°C following infection at 30°C, as described in Materials and Methods.
[c] Toto:*ts*118BC* contains the B fragment from *ts*118 and the C fragment from *ts*118R.

(Table 1). Thus, *ts*6 has one or more mutations in the region between *Spe*I (nt5262) and *Hpa*I (nt6919); if only a single mutation is involved, it must lie in the region of overlap of C1 and C2, between *Pst*I (nt5824) and *Hin*dIII (nt6267), which is located near the N terminus of nsP4.

For *ts*110, virus recovered from plasmid Toto:*ts*110C was *ts*, whereas virus from Toto:*ts*110A and Toto:*ts*110B was not. Plasmids Toto:*ts*110C1, Toto:*ts*110C2, and Toto:*ts*110C3 were then constructed and tested for fine mapping of the interval region C of *ts*110. *ts* viruses were obtained from recombinant plasmids Toto:*ts*110C2 and Toto:*ts*110C3, whereas plasmid Toto:*ts*110C1 gave rise to wild-type virus. From this we conclude that *ts*110 has one or more mutations in the region between nt5824 and nt7334 of the genome and that if a single mutation is involved, it must lie between nt6461 and nt6919 of the genome. This region is also in nsP4.

Mapping of *ts*118 suggested that it was a double mutant in which one mutation was located in the nsP4 region, as was the case for *ts*6 and *ts*110, and the second mutation was in a different region. Viruses from plasmids Toto:*ts*118A and Toto:*ts*118C were apparently wild type, whereas that from plasmid Toto:*ts*118B was partially *ts* (Tables 1 and 2). This partial temperature sensitivity manifested itself as a change in plaque size from small plaques at 30°C to minute plaques at 40°C, although the number of plaques at 30 and 40°C were the same. RNA synthesis at 40°C was reduced relative to that in Toto1101 virus (Table 2). When construct Toto:*ts*118BC was tested, the virus once again formed small plaques at 30°C, whereas at 40°C minute plaques were formed, with the plaque number being reduced by 4 orders of magnitude (Table 2), as was the case for the parental *ts*118. Furthermore, RNA synthesis at 40°C was reduced to very low levels (Table 2). Thus, we conclude that *ts*118 is a double mutant in which a mutation in the B region results in formation of minute plaques at 40°C but no change in plaque number and a second mutation in the C region, although having little apparent phenotype on its own, when combined with the change in the B region, results in reduction of plaque number (thus being scored as *ts* in plaque assays).

Fine mapping of the two *ts*118 mutations was done by constructing Toto:*ts*118B1, -B2, and -B3 (Fig. 1), and the change responsible for formation of minute plaques at 40°C mapped to region B1 (Table 1). Similarly, the change in the C region that, when combined with the B region change,

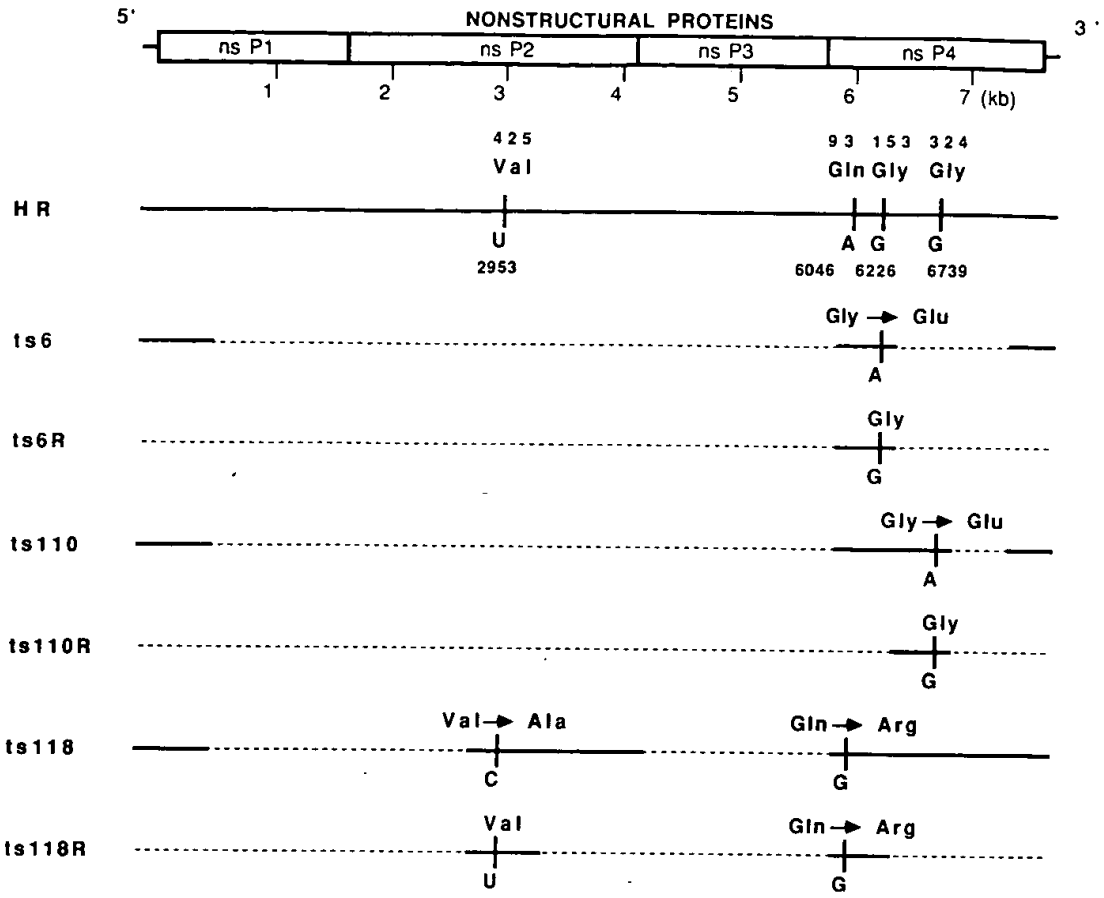

FIG. 2. Localization of *ts* mutations. A schematic of the nonstructural-protein-coding region of Sindbis virus is shown. Below are shown sequencing schematics for HR Sindbis virus (33), the parental strain from which the *ts* mutants were isolated (34), and for mutants *ts*6, *ts*110, and *ts*118 and their revertants. Sequenced regions are shown as solid lines. Any change from the HR sequence on the first line is indicated. Where no changes are shown, the sequence is identical to that of HR. Nucleotides are numbered from the 5' end of the RNA; amino acids are numbered from the N terminus of each protein.

resulted in reduction in plaque count was mapped to the region of overlap in C1 and C2 between nt5824 and nt6267.

Sequence analysis of *ts*6, *ts*110, and *ts*118 and their revertants. In order to define the *ts* lesions of *ts*6, *ts*110, and *ts*118, regions shown by the mapping experiments to contain the *ts* lesions were sequenced by the chemical method of Maxam and Gilbert (19) as modified by Smith and Calvo (29), with cDNA clones of mutants as well as of their revertants. The sequences obtained are shown in Fig. 2.

*ts*6 had a single base substitution in the region sequenced. Comparing the *ts*6 sequence with that of its revertant, and from the results in Table 1, we found that the mutation responsible for temperature sensitivity in *ts*6 was a change of G to A at nt6226, which led to the replacement of Gly (GGG) at position 153 of nsP4 by Glu (GAG). In the revertant, the changed nucleotide reverted to the original nucleotide, restoring the parental amino acid. *ts*110 also had only one change throughout the sequenced region. The change was G to A at nt6739, resulting in the change of Gly (GGG) to Glu (GAG) at position 324 of nsP4. In the *ts*110 revertant, this nucleotide reverted to the original nucleotide.

*ts*118 had a single base substitution in the region between nt2713 and nt3546. The change was U to C at nt2953, resulting in the change from Val (GUG) to Ala (GCG) at position 425 of nsP2. As discussed earlier, this change resulted in reduction in plaque size but not number at 40°C. In the *ts*118 revertant, this nucleotide reverted to the original nucleotide.

The second change in *ts*118 was found to be a change of A to G at position 6046, resulting in a Gln to Arg substitution at position 93 of nsP4. This change, combined with the change in nsP2, rendered the virus *ts* in that plaque number as well as plaque size were reduced at 40°C. The revertant selected, *ts*118R, retained the change in nsP4, which, as noted earlier, had little apparent phenotype on its own. An additional construct was made and tested in order to show that this change was in fact responsible in part for temperature sensitivity. The *ts*118R C region was combined with Toto:*ts*118B to produce Toto:*ts*118BC*, which upon assay was *ts*, forming tiny plaques at 40°C in reduced numbers (Table 2).

Characterization of the *ts* mutations rescued from *ts*6, *ts*110, and *ts*118. As a control to establish that the mutations mapped and defined here were the ones responsible for the phenotypes described previously for these mutations, and in order to establish the phenotype of these mutations in a uniform background, recombinant viruses containing a defined region from each of the *ts* mutants in a Toto1101 background were studied. Monolayers were transfected with dilutions of RNA transcribed from recombinant plasmids Toto:*ts*6C2, Toto:*ts*110C2, Toto:*ts*118B1, Toto:*ts*118C,

TABLE 3. Efficiency of plaque formation and RNA synthesis by *ts*6 and *ts*110 at 40 and 30°C[a]

Virus	Titer (PFU/ml)		Efficiency of plaquing (40°C/30°C)	Relative RNA synthesis		
	40°C	30°C		40°C	30°C	Shift to 40°C
Toto1101	5.2×10^8	2.0×10^9	2.6×10^{-1}	1.00	1.00	1.00
*ts*6	2.0×10^6	1.4×10^9	1.4×10^{-3}	0.04	0.38	0.02
Toto:*ts*6C2	2.7×10^5	8.0×10^9	3.4×10^{-5}	0.02	0.46	0.05
*ts*110	4.0×10^4	3.0×10^9	1.3×10^{-5}	0.05	0.72	0.16
Toto:*ts*110C2	1.2×10^4	2.1×10^9	5.7×10^{-6}	0.05	0.64	0.12

[a] See Table 2, footnotes *a* and *b*.

Toto:*ts*118BC, and Toto:*ts*118BC* and incubated at 30 and 40°C, with results similar to those shown in Table 1. A single plaque of each virus was isolated from the 30°C plate and a stock grown at 30°C, and these plaque-purified virus stocks were characterized further. To start, titers of these recombinant viruses were determined at 30 and 40°C to ascertain the relative efficiency of plaque formation, and the results are shown in Tables 2 and 3. The virus stocks derived from these infectious transcripts clearly showed the temperature sensitivity of the parental mutant in the case of *ts*6 and *ts*110, and the efficiency of plaque formation at 40°C relative to that at 30°C was low (Table 3) (the relatively high apparent reversion rate of *ts*6 is due to use of a stock that had not been recently plaque purified; during passage, revertants are amplified in most *ts* mutant stocks). However, as noted earlier, the virus recovered from Toto:*ts*118B1 formed minute plaques at 40°C, and the number of PFU was only slightly reduced (Table 2). The amino acid change of Val to Ala in nsP2 is thus responsible for the minute-plaque phenotype at the nonpermissive temperature but not for the reduced number of plaques. The mutation in nsP4 (construct Toto:*ts*118C) had little effect on its own, but in combination with the nsP2 change (constructs Toto:*ts*118BC and Toto:*ts*118BC*) it led to a pronounced decrease in the efficiency of plaque formation at 40°C (Table 2).

To examine these viruses further, RNA synthesis was analyzed after infection at 30°C, after infection at 40°C, and at 40°C following a shift from 30°C. The parental mutants *ts*6, *ts*110, and *ts*118 were included, as was virus recovered from clone Toto1101. Total viral RNA synthesis was analyzed by the cytoplasmic hybridization method of White and Bancroft (37) using ^{32}P-labeled minus-strand RNA transcribed from the structural protein region as a probe. The values determined, relative to those for Toto1101 virus, are shown in Tables 2 and 3. The synthesis of minus-strand RNA was significantly less than that of plus-strand RNA, and therefore the amount of plus-strand RNA detected by hybridization was assumed to be the total viral RNA. Following infection at 40°C, RNA synthesis by the viruses recovered from Toto:*ts*6C2 and Toto:*ts*110C2 was reduced to a level similar to that seen after infection by their respective parents. However, the virus recovered from Toto:*ts*118B1 showed a higher level of RNA synthesis than the parental *ts*118 at the nonpermissive temperature (Table 2). This is consistent with the observation that the mutation in nsP2 gives rise to minute plaques at 40°C but does not reduce plaquing efficiency. RNA synthesis by virus from Toto:*ts*118C, containing only the change in nsP4, was only slightly reduced at 40°C relative to that by Toto1101 virus and was the same as that by *ts*118R. However, RNA synthesis by the double mutants Toto:*ts*118BC and Toto:*ts*118BC* at 40°C was low and identical to that of the parental *ts*118, and thus the nsP4 mutation at position 93 markedly reduced RNA synthesis at the nonpermissive temperature when it was combined with the nsP2 change.

TABLE 4. Complementation analysis[a]

Virus	Complementation index					
	Group A (*ts*24)	Group B (*ts*11)	Group G (*ts*18)	Group F		
				*ts*6	*ts*110	*ts*118
Toto:*ts*6C2	53	48	55	0.4	—[b]	—
Toto:*ts*110C2	67	42	42	3	0.3	—
Toto:*ts*118B1	12	7	7	4	—	0.2
Toto:*ts*118BC	16	104	4	1	—	0.2

[a] Complementation indices shown are the yield from mixedly infected cells divided by the sum of the yields from singly infected cells. For *ts*118 constructs, the complementation indices are one way, as described in Materials and Methods.

[b] —, Not determined.

The mutants and the constructs were also tested for RNA synthesis after establishing infection at 30°C and shifting to 40°C, for comparison with the results of Keranen and Kaariainen (14) and Sawicki et al. (26) for *ts*6 (see also reference 2). *ts*6 makes very little RNA after a shift to 40°C, as found by Sawicki et al. (26), and the virus recovered from Toto:*ts*6C2 exhibited the same phenotype (Table 3). *ts*110 also made little RNA after a shift to 40°C, as did Toto:*ts*110C2 (Table 3). From a more detailed study of the kinetics of cessation of RNA synthesis in *ts*6-infected cells after a shift up (14, 26), and from studies of *ts*6 replication complexes in vitro (2), it was concluded that the elongation of RNA chains, as opposed to initiation, was *ts* in *ts*6, and it was postulated that the F group function, here shown to lie in nsP4, defined the RNA polymerase. The *ts*118 constructs such as Toto:*ts*118BC made significant amounts of RNA after a shift, much more RNA than when infection and incubation were done at 40°C continuously (Table 2). Thus, the replicase complexes of *ts*118, once formed at 30°C, are active after a shift to 40°C, in contrast to those specified by *ts*110 and *ts*6.

It is worth noting that the assay used for these shift experiments examines total virus plus-strand RNA in the infected cells at 8 h p.i. after a shift at 3.5 h. The results make clear that the RNA present at 3.5 h p.i. at 30°C does not contribute significantly to the RNA pool at 8 h, although the replicase enzymes needed for an essentially full yield of virus RNA are present. As a control for these results, we repeated the shift experiments and examined labeled RNA made between 3.5 and 8 h p.i. in the presence of ActD and [^{3}H]uridine. The results were similar to those presented in Tables 2 and 3.

Complementation analysis of rescued mutants. We also examined the ability of these viruses to complement representative *ts* mutants from the three other complementation groups of RNA$^-$ mutants. The complementation indices shown in Table 4 demonstrate that the viruses derived from Toto:*ts*6C2 and Toto:*ts*110C2 complemented the other three complementation groups of RNA$^-$ mutants, as did the parental viruses, but did not complement their parental viruses or each other, in agreement with previous complementation results (30).

Interpretation of results with virus derived from Toto:*ts*118B1 and Toto:*ts*118BC is complicated because of the high titer of virus produced at 40°C (the virus from Toto:*ts*118B1 was only marginally *ts*, as noted earlier, and virus

from Toto:*ts*118BC also leaked at 40°C compared with the other mutants studied). In these cases the complementation indices shown are one-way indices. Because viruses from Toto:*ts*118B1 and Toto:*ts*118BC formed minute plaques, it was possible to distinguish these plaques from those formed by the other *ts* mutants used. The complementation indices shown are the yield of large plaques in mixed infection divided by the yield of the large-plaque parents during single infection. Even so, complementation by Toto:*ts*118B1 was marginal, and we cannot assign it to a complementation group, although it did seem to complement all of the other mutants tried. Analysis of the double mutant Toto:*ts*118BC showed that it did not complement its parental virus or *ts*6 and only poorly complemented *ts*18 in group G, but complemented better the A mutant *ts*24 and quite well the B mutant *ts*11. Thus, except for the marginal complementation of Toto:*ts*118BC with *ts*18, the results with the rescued mutations are in good agreement with previous complementation results (30).

DISCUSSION

In this report we have localized the mutations responsible for the *ts* phenotype of complementation group F mutants. The mutations in *ts*6 and *ts*110 were mapped to nsP4. Each of them had a single base substitution, resulting in replacement of Gly by Glu at positions 153 and 324 of nsP4, respectively. Analysis of these mutations in a Toto1101 background in a variety of ways, including RNA synthesis at 40°C and ability to complement other RNA^- mutants, demonstrated that these mutations are in fact those responsible for the *ts* phenotype and the mutant phenotypes previously described in the literature. *ts*118 turned out to be a double mutant. It had a defect in nsP2 (a Val to Ala change at position 425) which only partially disabled it at 40°C, resulting in production of small plaques and reduced RNA synthesis, although the plaque number was unchanged. A second mutation in nsP4 combined with this change in nsP2 resulted in true temperature sensitivity, in that the plaque titer as well as plaque size was reduced at 40°C and RNA synthesis after infection at 40°C was reduced to the level characteristic of RNA^- mutants. It is of interest that this change in nsP4 alone had little apparent phenotype, but because it is the one responsible for temperature sensitivity, leading to reduced plaque titer in combination with the nsP2 change, the double mutant *ts*118 complements *ts*6 and *ts*110 as a group F mutant. Thus, mutations in nsP4 lead to group F complementation behavior.

The experiments of Fuller and Marcus (8) in which the complementation groups were ordered by the relative rate of UV inactivation of their ability to complement gave an order for the complementation groups of NH_2-G-A-B/F-COOH. Mutations in B and F could not be precisely localized, but both occurred downstream of group G and group A. Thus, the UV mapping data are consistent with our results that group F mutants contain defects in nsP4. However, mapping of the other complementation groups of Sindbis virus *ts* RNA^- mutants, currently being carried out, indicates that the Fuller and Marcus (8) order is not correct (Y. S. Hahn, unpublished observations).

Keranen and Kaariainen (14) and Sawicki et al. (26) demonstrated that *ts*6 ceased genomic, subgenomic, and minus-strand RNA synthesis upon a shift from the permissive to nonpermissive temperature and postulated that there was a *ts* lesion in the elongation component of the replicase. Recently, Barton et al. (2) extended such studies to in vitro studies of replication complexes isolated from *ts*6-infected cells and concluded that such transcription complexes were *ts* in elongation. In contrast, other mutants examined retain activity at 40°C if replication complexes are formed first at 30°C (14). These results, together with the results presented here, suggest that nsP4 is an RNA polymerase or the elongation component of the alphavirus replicase. In this regard, it is of considerable interest that nsP4 contains the Gly-Asp-Asp sequence and surrounding hydrophobic amino acids that have been found to be present in the replicase proteins of several other RNA viruses (13, 23).

```
      ns P4                 E:ts6
                              ↓                170
SIN   NYPTVASYQITDEYDAYLDMVDGTVACLDTATFCPAKLRS
SF    -----------------------SDS---R---------C
RR    -----------------------SES---R---------C
ONN   ---------V-------------SES---R---N-S----
MID   -----T-----------------SES---R-A---S----

      ns P4                      E:ts110
                                   ↓           336
SIN   PGTKHTEERPKVQVIQAAEPLATAYLCGIHRELVRRLTAV
SF    -------------------------------------N--
RR    -------------------------------------K--
ONN   -------------------------------------N--
MID   -------------------------------------N--

      ns P2                      A:ts118
                                   ↓           437
SIN   QKVNENPLYAITSEHVNVLLTRTEDRLVWKTLQGDPWIKQ
SF    ----------PA--------------------A------V
RR    ----------PS------------N-------S------V
ONN   ----------P-------------GK-T----S------I

      ns P4                    R:ts118
                                 ↓             106
SIN   KVENQKAITTERLLSGLRLYNSATDQPEC YKITYPKPLY
SF    ----M--TVVD--T--A---TG-DVGRIPT-AVR--R-V-
RR    ----M--VIID--KD-A-T-LTEQSEKIPT-VSK--R-V-
ONN   ----M--TIIH--KE-C---LASETPRVPS-RVT--A-I-
MID   ----M--EVID--LG-AK-FVTP-TDCRY VTHKH---M-
```

FIG. 3. Amino acid sequences of proteins from five alphaviruses in the region near the group F mutations. Sequence data are from the following sources: Sindbis virus (SIN), Strauss et al. (33); Semliki Forest virus (SF), Takkinen (36); Ross River virus (RR), Faragher et al. (7) and Strauss et al. (31); O'Nyong-nyong virus (ONN), Strauss et al. (31) and unpublished data; Middelburg virus (MID), Strauss et al. (32).

The mutations in *ts*6, *ts*110, and *ts*118 are shown in Fig. 3, in which the sequences of up to five alphaviruses are compared in the regions affected. It is readily evident that the glycines affected in nsP4 of *ts*6 and *ts*110 are conserved in all alphaviruses sequenced to date and that each is found within a long stretch of highly conserved amino acids. In view of this conservation in sequence, it is evident that changes in the sequence, such as in *ts*6 and *ts*110, might affect function. The nsP4 polypeptide is composed of 610 amino acids and is on average 71 to 74% conserved among alphaviruses which have been examined (31). However, this conservation is not uniform throughout the protein; amino acids 1 to 125 from the N terminus (including the location of the change in *ts*118) are less highly conserved, as are amino

acids 550 to 604 near the C terminus. The canonical sequence Gly-Asp-Asp, flanked by hydrophobic amino acids, is found at residues 464 to 466. This sequence, which has been found in a number of RNA-dependent RNA replicases (13; see also discussion in reference 23), is well separated (on the linear sequence) from either the *ts*6 or *ts*110 lesion.

The change of Val to Ala in nsP2 of *ts*118 is found in a domain that is well conserved among alphaviruses, although conservation is not absolute. In particular, the Val affected in *ts*118 is not totally conserved, being replaced by Thr in O'Nyong-nyong virus. The Gln to Arg change in nsP4 of *ts*118 affects an amino acid in a domain that is not conserved (Fig. 3). The Gln is replaced by Gly, Glu, Pro, or Asp in different alphaviruses. Thus, the failure of this change to have much effect by itself is perhaps not surprising. The pronounced effect of this substitution in combination with the change in nsP2 suggests that nsP2 and nsP4 may interact to form a functional complex, although other explanations for such a synergistic effect are possible.

In Sindbis virus, translation of nsP4 requires readthrough of an opal termination codon so that nsP4 is underproduced relative to nsP1, nsP2, and nsP3 (11, 16, 32). The active form of nsP4 may be the polypeptide nsP34, which accumulates during Sindbis virus infection, whereas little or no nsP4 is detected. The finding that nsP4 may be the viral RNA polymerase, based on results with complementation group F mutants and the presence of motifs within this protein that are shared with other RNA polymerases of animal viruses (13), is then reminiscent of the control of virus replicases in other systems in which readthrough of a termination codon is required to produce the polymerase. In tobacco mosaic virus, readthrough of an amber codon is required (9, 21), and the readthrough portion of the protein is homologous to that of the Sindbis virus protein (12). Similarly, in the retroviruses, translation of the reverse transcriptase requires readthrough of an amber codon or frame shifting to eliminate an amber codon (25, 27, 28). We presume that regulation of the amount of RNA polymerase produced is important during the viral life cycle. As has been pointed out, however, Semliki Forest virus and O'Nyong-nyong virus lack this termination codon (31, 36), and regulation of the activity of the polymerase seems to be different for these alphaviruses.

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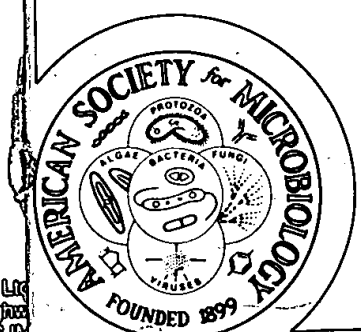

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